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Enhanced Phospholipase C Activity in the Cultured Skin Fibroblast Obtained from Patients With Coronary Spastic Angina: Possible Role for Enhanced Vasoconstrictor Response

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OBJECTIVES We measured phospholipase C (PLC) activity in the cultured skin fibroblasts obtained from patients with and without coronary spasm and examined its correlation with coronary artery vasomotility.

BACKGROUND Coronary artery vasomotility is enhanced in coronary spastic angina (CSA), but no information is available for the intracellular signaling. In spontaneously hypertensive rats, PLC activity in the skin fibroblasts has been shown to be enhanced.

METHODS Skin fibroblasts obtained from 24 patients with CSA—14 with organic coronary artery disease (CAD) and 12 control subjects—were cultured by the explant method. Activity of PLC was determined by incubating the membrane fraction with ^{3}H-phosphatidyl inositol bisphosphate and by quantifying ^{3}H-inositol trisphosphate. In patients with CSA and control subjects, the relations between PLC activity and coronary artery basal tone and constrictor response to intracoronary acetylcholine (ACh) were examined.

RESULTS Activity of PLC (pmol/protein [mg] per min) was 1.74 ± 0.19 in patients with CSA; 0.90 ± 0.12 in patients with CAD; and 0.65 ± 0.07 in control subjects ($p < 0.001$, patients with CSA vs. patients with CAD and control subjects; p = NS, patients with CAD vs. control subjects). According to the Lineweaver-Burk plot, Michaelis constant (μmol/liter) of PLC was 28 ± 4 in patients with CSA; 49 ± 14 in patients with CAD; and 56 ± 10 in control subjects ($p < 0.05$, patients with CSA vs. control subjects), whereas the maximal velocity was not different between the three groups. There were significant positive correlations between PLC activity and both basal tone ($p = 0.0108$) and response to ACh ($p = 0.0053$). Western blot analysis using membrane fraction demonstrated that 89% of PLC isoenzymes detected was of the δ1 isoform.

CONCLUSIONS Because the PLC activity measured was genetically defined and was positively correlated with coronary artery vasomotility, enhanced PLC activity may be involved in the pathogenesis of coronary spasm. (J Am Coll Cardiol 2000;36:1847–52) © 2000 by the American College of Cardiology

Coronary artery spasm plays an important role in the pathogenesis of variant angina (1–3), as well as in other acute coronary syndromes (4,5). We (6) and other investigators (7,8) have shown that the basal tone of the entire coronary artery system of Japanese patients with variant angina is enhanced. Occlusive coronary artery constriction is readily induced by diverse constrictor stimuli (9). These suggest an impaired intracellular signaling in the process of coronary artery smooth muscle contraction.

Phospholipase C (PLC) hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP_2) and produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. The IP_3 mobilizes Ca^{2+} from the intracellular stores and elicits rapid contraction of the smooth muscle cells (10). Diacylglycerol activates protein kinase C (PKC) and initiates sustained contraction (11). Using swine coronary spasm models, Ito et al. (12) and Katsumata et al. (13) indicated that the PKC-mediated pathway and enhanced myosin light chain phosphorylation play an important role in the enhanced constrictor response of the coronary artery smooth muscle. In contrast, PLC activity has been shown to be enhanced not only in the vascular smooth muscle cells (14), but also in the fibroblasts obtained from the aortic adventitia (15) and skin (16) of spontaneously hypertensive rats. This enhancement is present while the rats are in the prehypertensive state (14). The enhanced PLC activity in the fibroblasts may be linked with that in the vascular smooth muscle cell, because these

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Abbreviations and Acronyms

ACh = acetylcholine
CAD = coronary artery disease
CSA = coronary spastic angina
IP_3 = inositol 1,4,5-trisphosphate
ISDN = isosorbide dinitrate
K_m = Michaelis constant
PIP_2 = phosphatidyl inositol 4,5-bisphosphate
PKC = protein kinase C
PLC = phospholipase C
V_{max} = maximal velocity of reaction

cells have similar characteristics as connective tissue cells (17,18).

In clinical cases of variant angina, the incidences of both migraine and Raynaud's phenomenon were high as compared with those in control subjects (19). Also, enhanced esophageal motility in patients with variant angina has been reported (20). These suggest a generalized disorder of smooth muscle contraction and led us to test the hypothesis that impaired intracellular signaling is present in these patients. The present study was designed to examine PLC activity in the cultured skin fibroblasts obtained from the patients with coronary artery spasm and to compare it with that in the patients without coronary spasm. Skin fibroblast was used because it can be readily obtained and is suitable for the experiments on cultured cells.

METHODS

Study patients. The study protocol was approved by our Institution's Ethics Committee. Written, informed consent was obtained from all patients before the study. Fifty patients in whom the skin fibroblast was successfully cultured were included in the study. Group A consisted of 24 patients with coronary spastic angina (CSA) (Table 1) and without significant stenotic coronary artery lesions. Ten of the 24 patients had angiographically normal coronary arteries, whereas the remaining 14 had mild (≤50% lumen diameter stenosis) atherosclerotic lesions. In 12 patients, ST segment elevation was recorded on the electrocardiogram (ECG) during a spontaneous or hyperventilation-induced attack. Coronary spasm, defined as total occlusion or severe vasoconstriction of the coronary artery associated with chest pain and ischemic ECG changes, was induced with an intracoronary injection of acetylcholine (ACh) in 20 patients. In the remaining four patients, all of whom had been diagnosed with variant angina, induction of spasm was not attempted. Group B consisted of 14 patients with significant coronary artery disease (CAD). None had a clinical history suggestive of CSA. Group C consisted of 12 control subjects without angina pectoris and with normal coronary arteries. These patients underwent cardiac catheterization because of atypical chest pain (n = 8) or cardiac arrhythmias (n = 4). Treadmill exercise and hyperventilation tests were performed in the patients with atypical chest pain, and neither anginal attack nor ST segment change was induced in any of them. Intracoronary ACh was done in six of them, and coronary spasm was not induced in any patients.

Table 1. Clinical Profiles of the Study Patients

	Group A (n = 24)	Group B (n = 14)	Group C (n = 12)
Sex (male/female)	19/5	11/3	6/6
Age (yr)	62 ± 2	66 ± 2	61 ± 2
Smoking	16/24 (67%)	9/14 (64%)	6/12 (50%)
Systolic blood pressure (mm Hg)	117 ± 4	122 ± 6	127 ± 4
Diastolic blood pressure (mm Hg)	71 ± 2	71 ± 3	77 ± 4
Total cholesterol (mg/dl)	193 ± 6	192 ± 11	187 ± 9
LDL cholesterol (mg/dl)	110 ± 5	121 ± 9	111 ± 5
HDL cholesterol (mg/dl)	54 ± 2	45 ± 4	52 ± 5
Triglyceride (mg/dl)	144 ± 15	133 ± 19	121 ± 19
Blood sugar (mg/dl)	100 ± 4	116 ± 6*	101 ± 3
BMI (kg/m^2)	22.7 ± 0.5	22.9 ± 0.6	24.2 ± 1.0

BMI = body mass index; HDL = high density lipoprotein; LDL = low density lipoprotein. Values are mean ± SEM. *$p < 0.05$ vs. Groups A and C.

In all three groups, patients with hypertension (systolic pressure ≥160 mm Hg or diastolic pressure ≥95 mm Hg) or who had received medication for hypertension in the past were excluded from the study, because hypertension itself may affect PLC activity (14). In Groups A and C, all medications except sublingual nitroglycerin were withdrawn at least three days before the angiographic study.

Cardiac catheterization. The coronary arteriographic study was performed while the patients were in the fasting state. Eleven ECG leads, except aVR, and arterial pressure were continuously monitored during catheterization. In 20 patients in Group A and six in Group C with atypical chest pain, after baseline coronary arteriography, an intracoronary injection of ACh (50 and 100 μg) was given (21). In two patients in Group A, ACh was injected only in the right coronary artery. When ACh-induced spasm did not resolve spontaneously within 2 min or if hemodynamic instability developed, isosorbide dinitrate (ISDN) was administered. Coronary arteriograms were taken in multiple projections after intracoronary ISDN (2.0 mg).

Quantitative coronary angiography and analysis of response to ACh. Each of the three major coronary arteries was divided into three or four segments, and the lumen diameter of each segment was measured quantitatively (Cardio 500, Kontron Electronik, Munich, Germany). In Group A, the measurement was done at baseline, after intracoronary ACh-induced coronary spasm and after ISDN. In Group C, it was done at baseline, after intracoronary ACh (100 μg for the left and 50 μg for the right coronary artery) and after ISDN. The basal tone was calculated as follows: 100 × (diameter after ISDN − baseline diameter)/diameter after ISDN. The response to ACh was calculated as follows: 100 × (baseline diameter − diameter after ACh)/baseline diameter. Because the patients with coronary spasm have enhanced basal tone (6,8), the response to ACh was normalized by the diameter after

ISDN and calculated as follows: 100 × (diameter after ISDN − diameter after ACh)/diameter after ISDN. Both the basal tone and response to ACh were calculated for all coronary artery segments. The average basal tone and maximal constrictor response and average response to ACh were determined for each patient.

Culture of skin fibroblast. At the time of cardiac catheterization, a small piece of skin was obtained from the femoral region. Primary fibroblasts were cultured by the explant method (22). Fibroblasts from the second to fourth passages were used for the study. Our preliminary study revealed that there was no difference in PLC activity (pmol/protein [mg] per min) among fibroblasts obtained from different regions (0.93 ± 0.16 in the cervical and 0.92 ± 0.17 in the femoral regions, n = 6) and among fibroblasts at different passages (0.87 ± 0.20 at the second and 0.93 ± 0.19 at the fourth passages, n = 4).

Membrane preparation. Confluent monolayers were scraped and homogenized (23). The homogenate was centrifuged at 500 *g* for 10 min, and the supernatant was centrifuged at 40,000 *g* for 15 min. The pellet was stored at −80°C. The protein content was measured spectrophotometrically.

Assay for membrane PLC activity. The PLC assay system included the following components (24): N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (50 mmol/liter), $CaCl_2$ (0.1 mmol/liter), sodium cholate (9 mmol/liter), ^{3}H-PIP_2 (40,000 cpm) and membrane protein (20 μg), in a final volume of 200 μl. Four assay systems were incubated for 0, 2, 4 and 6 min at 37°C. The reaction was stopped with chloroform/methanol/hydrogen chloride (HCl) followed by 1N HCl containing EGTA. After extraction, the aqueous phase was removed for liquid scintillation counting. Production of ^{3}H-IP_3 was linear when the reaction time was between 0 and 6 min and membrane protein was between 10 and 40 μg.

Michaelis constant (K_m) and maximal velocity (V_{max}) of PLC. In 22 patients in Group A, 10 patients in Group B and 9 patients in Group C, PLC activity was determined under high concentrations of cold PIP_2 (50, 25, 12.5 and 6.25 μmol/liter), and a Lineweaver-Burk plot was delineated. The K_m, which reflects the affinity of PLC for substrate, with a low K_m indicating high affinity, and V_{max}, which reflects the amount of the enzyme, were calculated according to this plot.

Western blot analysis of PLC isoenzymes. Membrane fraction obtained from four patients in Group A and four patients in Group C was subjected to SDS-PAGE using a gradient gel. Protein was transferred electrophoretically to a nitrocellulose membrane. The membranes were then treated with anti-PLC-β2 and β3 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, California) and anti-PLC-γ and δ1 monoclonal antibodies (Transduction Laboratories, Lexington, Kentucky) and stained by amplified alkaline phosphatase immunoblot kits.

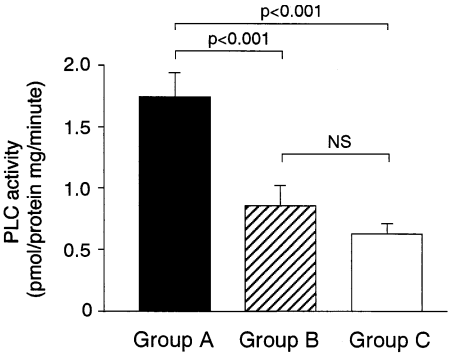

Figure 1. Mean phospholipase C activity of the three groups. See text for details.

Data analysis. All data are shown as the mean value ± SEM. Comparison of continuous variables among the three groups was performed with one-way analysis of variance followed by the Fisher protected least significant difference multiple comparisons test, and comparison of categoric variables was done using the chi-square test. To examine whether any of these variables had an effect on PLC activity, univariate analysis was performed. The interaction between the variables and PLC activity was examined by multiple regression analysis. The relations between each of the average basal coronary artery tone, maximal constrictor response to ACh and average response to ACh and PLC activity were examined with linear regression analysis. A p value <0.05 was considered statistically significant.

RESULTS

Clinical profiles of the patients. Table 1 shows the clinical profiles and laboratory data of the study patients. There was no statistical difference among the three groups, except for fasting blood sugar level. When the variables were compared between Group A and Groups B and C, none of them was different between the groups.

Comparison of PLC activity, K_m and V_{max}. Figure 1 shows PLC activity for the three groups. Mean PLC activity (pmol/protein [mg] per min) was 1.74 ± 0.19 in Group A, 0.90 ± 0.12 in Group B and 0.65 ± 0.07 in Group C ($p < 0.001$, Group A vs. Groups B and C; p = NS, Group B vs. Group C). In Group A, there was no statistical difference in the activity between the patients with angiographically normal coronary arteries (1.81 ± 0.23) and those with mild coronary artery disease (1.57 ± 0.34). Mean K_m (μmol/liter) in Groups A, B and C were 28 ± 4, 49 ± 14 and 56 ± 11, respectively (p = 0.023, Group A vs. C; p = 0.0701, Group A vs. B), whereas mean V_{max} (pmol/protein [mg] per min) were 941 ± 182, 592 ± 97 and 755 ± 140, respectively (p = NS, among the three groups).

Analysis of the effect of clinical profiles on PLC activity. Univariate analysis showed that among the clinical variables listed in Table 1, only age was correlated with PLC activity ($y = 2.91 - 0.03x$, $R^2 = 0.08$, p = 0.045). Multiple

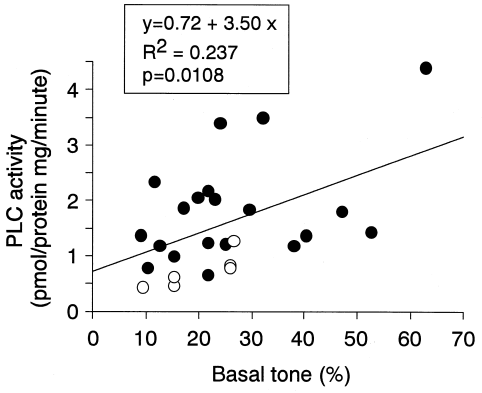

Figure 2. Relationship between the average basal coronary artery tone and PLC activity. **Open circles** = control subjects without coronary spasm; **solid circles** = patients with CSA. See text for details.

regression analysis, however, revealed that none of the variables was significantly correlated with PLC activity.

Relationship of PLC activity with coronary artery vasomotility. BASAL TONE. The average coronary artery diameter after ISDN was not different between Group A (2.8 ± 0.1 mm) and Group C (2.7 ± 0.1 mm). The average basal tone was 26.8 ± 3.3% in Group A and 19.7 ± 3.0% in Group C (p = NS). A significant positive correlation was noted between the average basal coronary artery tone and PLC activity (Fig. 2).

RESPONSE TO ACH (FIG. 3). The maximal constrictor response to ACh was 82.3 ± 3.6% in Group A and 39.2 ± 3.1% in Group C ($p < 0.0001$). A significant positive correlation was noted between the maximal constrictor response to ACh and PLC activity (Fig. 3A) and between that normalized by ISDN and PLC activity (Fig. 3B).

The average response to ACh was 45.0 ± 2.0% in Group A and 14.5 ± 1.6% in Group C ($p < 0.0001$). A significant positive correlation was noted between the average response to ACh and PLC activity (Fig. 3C) and between that normalized by ISDN and PLC activity (Fig. 3D).

Phospholipase C isoenzymes in membrane fraction of skin fibroblast. Western blot analysis demonstrated that 88.7 ± 3.4% of PLC isoenzymes detected was of the δ1 isoform and the other small fraction β3. Figure 4 shows PLC-δ1 consisting of 70- and 85-kd bands for two patients in Group A (lanes 3 and 4) and two patients in Group C (lanes 1 and 2). The ratio of these bands to bovine serum albumin as a molecular standard was not different between Group A (0.66 ± 0.12, n = 4) and Group C (0.69 ± 0.09, n = 4).

DISCUSSION

A diffuse vasomotion abnormality in all of the epicardial coronary arteries has been suggested to be involved in the pathogenesis of coronary spasm, at least in Japanese patients (6–8,25). Maseri et al. (26), however, described that the cause and significance of the generalized increase in coronary vasomotility seen in some patients with variant angina differ from those of segmental occlusive spasm, and they

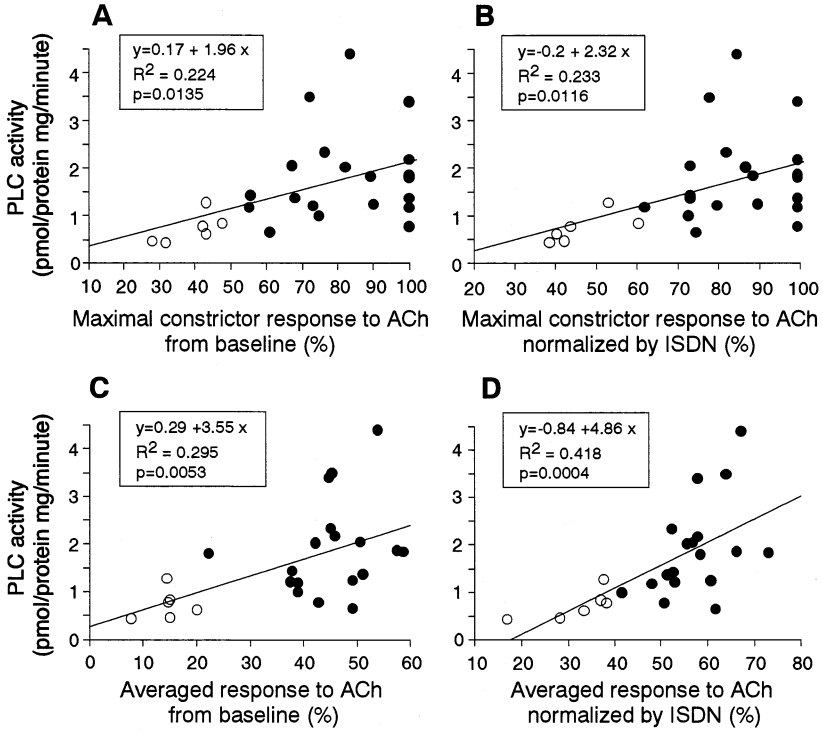

Figure 3. Relations between the maximal constrictor response to ACh from the baseline diameter and PLC activity (**A**), between the maximal constrictor response normalized by ISDN and PLC activity (**B**), between the average response to ACh from the baseline diameter and PLC activity (**C**) and between the average response normalized by ISDN and PLC activity (**D**). **Open circles** = control subjects without coronary spasm; **solid circles** = patients with CSA. See text for details.

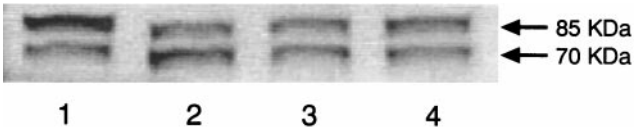

Figure 4. Western blot analysis of the membrane fraction, demonstrating the phospholipase C-δ1 isoenzyme stained as 70- and 85-kd (kDa) bands. **Lanes 1 and 2** = control subjects; **lanes 3 and 4** = patients with CSA.

recently pointed out the racial differences in the coronary vasomotor reactivity between Japanese and white patients (27).

The endothelium-dependent vasodilation to ACh was shown to be impaired during the atherosclerotic process (28). A diffuse vasomotion abnormality in CSA, however, cannot be explained solely by the atherosclerotic process that causes a "paradoxic" vasoconstriction to ACh (29). Recently, Nakayama et al. (30) reported that the $T^{-786} \rightarrow C$ mutation in the endothelial nitric oxide synthase gene reduces nitric oxide synthesis and may predispose the patients with this mutation to coronary spasm. However, all of the patients do not necessarily have this gene mutation.

Enhanced PLC activity in patients with coronary spasm. The initial intracellular signaling for vascular smooth muscle contraction involves hydrolysis of PIP_2 in the plasma membrane with activated PLC, and the resultant production of IP_3 and diacylglycerol. In spontaneously hypertensive rats, PLC activity has been shown to be enhanced not only in the vascular smooth muscle cells (14), but also in the fibroblasts obtained from the aortic adventitia (15) and skin (16). The enhanced PLC activity, genetically defined, may possibly induce pathologic changes in the blood vessels and may cause physiologic disorders in the regulation of systemic and regional circulation.

The present study demonstrated that PLC activity in the skin fibroblast was enhanced in patients with CSA. Our preliminary study showed that the stimulation of the membrane fraction of cultured skin fibroblasts with arginine-vasopressin or angiotensin II did not affect PLC activity. Thus, the enhanced PLC activity is suggested to be due to the impairment of PLC itself or its regulatory mechanism. This study further revealed that K_m, a concentration of substrate at which half of V_{max} was obtained, was significantly smaller in the group of patients with CSA than in the other groups, whereas V_{max} was not different between the patients with and without coronary spasm. This suggests that the enhanced PLC activity was not due to a quantitative impairment, but rather to a qualitative impairment. Although patients with hypertension were excluded, the blood pressure in the patients with enhanced PLC activity (Group A) was not high as compared with that in the patients with lower activity (Groups B and C). Also, there was no significant correlation between PLC activity and blood pressure. Enhanced PLC activity, as shown in this study, may not be linked with the pathogenesis of hypertension.

At present, there have been 10 PLC isoenzymes identified, excluding alternatively spliced forms (31). Four isoenzymes of PLC-β, two of PLC-γ and four of PLC-δ have been reported. The present Western blot analysis demonstrated that a major PLC isoenzyme detected in the membrane fraction of skin fibroblast was of the δ1 isoform, and there was no difference in the amount between Groups A and C. It was recently reported that the missense mutation in the PH domain of the human PLC-δ1 gene was associated with a remarkable loss of function (32). Further studies on the mechanisms of enhanced PLC-δ1 activity in the patients with CSA should be required.

Relationship between PLC activity and coronary vasomotility. The constrictor response to ACh of the coronary arteries of the patients with CSA was enhanced, as compared with that of the patients without spastic angina. In contrast, the basal coronary artery tone in the patients with coronary spasm was not increased, which is inconsistent with the findings of our previous report (6). The coronary angiographic study in the previous study was performed in the early morning, whereas that in the present study was not necessarily done in the morning.

To clarify the relationship between PLC activity determined in the skin fibroblast and coronary artery vasomotility, we examined how PLC activity was correlated with the coronary artery basal tone and vasomotor response to ACh. It was clearly shown that PLC activity was correlated significantly with not only basal coronary artery tone, but also the maximal constrictor and average responses of the coronary artery to ACh. Thus, higher PLC activity corresponded with greater basal tone and constrictor response to ACh. This correlation of PLC activity with coronary artery vasomotility may suggest the role of enhanced PLC activity in the genesis of coronary spasm.

Study limitations and implications. The major limitation of this study was the lack of evidence that PLC activities of the skin fibroblast and coronary artery smooth muscle cell are closely correlated with each other. It is almost impossible to obtain the coronary artery smooth muscle cell from patients with CSA. Furthermore, at present, it is impossible to estimate PLC activity of the coronary artery smooth muscle in situ. We used cultured fibroblast, and the PLC activity that was determined was most likely to be genetically defined. The correlation between measured PLC activity and coronary artery vasomotility determined in vivo may be indirect evidence for the role of enhanced PLC in the genesis of coronary spasm; further studies are required.

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